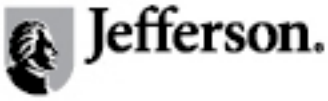



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# Advances in Chimeric Antigen Receptor T-Cell Therapies for Solid Tumors.

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**State of the Art**

# Advances in Chimeric Antigen Receptor (CAR)-T Cell Therapies for Solid Tumors

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**ABSTRACT**

In 2017, the United States Food and Drug Administration (FDA) approved the first two novel cellular immunotherapies using synthetic, engineered receptors known as chimeric antigen receptors (CAR), tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta), expressed by patient-derived T cells for the treatment of hematological malignancies expressing the B-cell surface antigen CD19 in both pediatric and adult patients. This approval marked a major milestone in the use of antigen-directed 'living drugs' for the treatment of relapsed or refractory blood cancers, and with these two approvals, there is increased impetus to expand not only the target antigens but also the tumor types that can be targeted. This state-of-the-art review will focus on the challenges, advances, and novel approaches being utilized to implement CAR-T cell immunotherapy for the treatment of solid tumors.

## INTRODUCTION

The human T-cell pool can be broadly separated into two populations with distinct functional roles based on the surface antigens CD4 and CD8. The $CD4^+$ T cells, also known as helper T cells, coordinate and regulate immune responses, whereas the $CD8^+$ T cells, or cytolytic T cells, kill cells expressing a defined target antigen. Both $CD4^+$ and $CD8^+$ T-cell function is dependent on the binding of a highly variable T-cell receptor (TCR) to its cognate antigenic peptide presented by major histocompatibility complex (MHC) molecules which are highly polymorphic within patients. For TCRs in $CD8^+$ T cells, the corresponding MHC class I molecule is expressed by all cells in the body, including tumor cells. It is this $CD8^+$ T-cell response that is critical in cancer immunotherapy. Indeed, patients with various solid tumors have an improved prognosis when endogenous, cytolytic $CD8^+$ T cells are present within the tumor (1).

These tumor-resident T cells, or tumor infiltrating lymphocytes (TILs), can be obtained from the patient, selected for tumor specificity and expanded *ex vivo*, and reinfused into the patient as a highly personalized treatment for multiple solid tumor types (2,3). Moreover, known tumor antigen-specific TCRs have been cloned and genetically engineered into T cells for adoptive transfer; however, the efficacy of these engineered TCRs is restricted by the highly variable human leukocyte antigen (HLA) haplotypes, which encode the MHC molecule, limiting broad applicability across the patient population.

Infusing naturally-occurring or TCR-engineered tumor-specific T cells into patients has been therapeutically successful; however, there are several limitations to this approach. In a cohort of nine patients with tumors expressing the melanoma-associated antigen A3 (MAGE-A3), clinical responses, as defined by RECIST criteria, were observed in 5 patients [partial responses (PR) =4;

complete responses (CR)=1] (4). Severe neurological toxicity was observed in three patients, although it is unclear if the toxicity was due to concomitant cytokine administration or off-target effects or, more likely, a combination of specific and indirect inflammatory events. While the engineered TCR was selected because of its recognition of MAGE-A3, it was discovered by the authors that this TCR also recognizes MAGE-A12, MAGE-A2, and MAGE-A6. Biochemical analyses revealed expression of several members of the MAGE-A gene family, including A3, A6, and A12, in the brain tissue of patients with observed neurological toxicity and recognition of MAGE-A12 was identified as the most likely cause of toxicity. In a separate case, an affinity-enhanced MAGE-A3 TCR was developed, and therapeutic use resulted in lethal cardiotoxicity, later attributed to TCR cross-reactivity with an epitope in the cardiac protein, titin (5,6).

In a single case study of a patient with metastatic colorectal cancer, tumor-specific $CD8^+$ T cells recognizing mutant KRAS G12D were expanded *ex vivo* and reinfused into the patient who harbored seven lesions in the lung. Initially, all seven lesions responded to treatment at six weeks; however, one lesion eventually progressed, and it was discovered after resection that this lesion lost expression of the MHC molecule presenting the KRAS G12D epitope, evading the infused, tumor-specific T cells (7). In a letter to the editor regarding that study, it was noted that the probabilistic occurrence of a patient having both a KRAS G12D mutation and the appropriate HLA haplotype to express the mutant peptide epitope is exceedingly rare (8). In a survey of the Cancer Genome Atlas the authors found in their sample size of 6125 patients that 151 were positive for the KRAS G12D mutation and of that population, only 4 had the corresponding HLA allele. While TCR-based immunotherapy is efficacious, its highly-personalized nature limits its feasibility as a future standard-of-care therapy, especially with the prospect of MHC loss by the tumor and off-

target toxicity risks due to a nearly unknowable immuno-peptidome. In contrast, CAR-T cells can bridge this gap and provide a therapy that offers robust, targeted antitumor efficacy that can be more broadly applied to the patient population.

Developed in the late 1980s by Zelig Eshhar of the Weismann Institute in Israel, chimeric antigen receptors (CARs) are synthetic receptors that pair the epitope binding domains of an antibody with the intracellular signaling domains of a T-cell receptor (9). By targeting epitopes in their native form, CARs can function independently from peptide-MHC presentation, executing the same cytotoxic effector function as endogenous $CD8^+$ T cells (**Fig. 1**). Since their inception, CARs have evolved through three generations of development. The first generation consisted of the antigen-binding domain of antibodies, known as a single chain variable fragment (scFv), which is a fusion of the heavy and light chain variable regions with a flexible peptide linker, and the intracellular domains of the signaling protein CD3ζ of the TCR complex. These CARs could not persist *in vivo*, and it was discovered that inclusion of signaling domains from co-stimulatory molecules, such as CD28, could enhance survival of CAR-T cells expressing these second-generation CARs (10). Third-generation CARs include domains from two co-stimulatory molecules, such as CD28 and 4-1BB (CD137). The two FDA-approved CAR-T cell therapies are based on second-generation designs, with Kymriah using 4-1BB as the co-stimulatory domain, and Yescarta using CD28. Both designs are effective in the treatment of hematological malignancies; however, there is evidence to suggest that strategic deployment of both designs may be required for therapeutic efficacy in solid tumors.

**OVERCOMING THE SOLID TUMOR BARRIER**

The excitement surrounding CAR-T cell immunotherapy stems from the successful treatment of numerous relapsed and refractory hematological malignancies expressing the surface antigen CD19, including acute (11,12) and chronic (13,14) lymphocytic leukemia (ALL and CLL), lymphoma (15), and diffuse large B-cell lymphoma (16,17). To date, the successful implementation of CAR-T cells in the treatment of hematological malignancies has not extended to solid tumors (**Fig. 2**). The greatest challenge to implementing successful CAR-T cell therapy for solid tumors is finding the appropriate target antigen. While CD19-directed CAR-T cells do exhibit on-target, off-tumor toxicity, the resulting B-cell aplasia, while lasting for at least six months (18), can be successfully managed with intravenous pooled immunoglobulin (IVIG). Many antigens that are present in solid tumors are often also present in the normal tissue of origin. Whereas B-cell aplasia can be managed clinically, destruction of vital organs is treatment-limiting and potentially fatal. While robust human data regarding the safety of CAR-T cells is not currently available for solid malignancies, it should be noted that engineered TCRs and CAR-T cells directed towards the same antigen may have vastly different safety profiles due to spatial differences in native antigen expression versus MHC class I presentation to T cells and differences in antigen cross-reactivity profiles.

Although trafficking of CAR-T cells to tumors is an important concern regarding efficacy (19), solid tumor treatment may benefit from local infusion at the site of metastases, rather than systemic administration (20). More important, once the cells arrive at the tumor, will be the ability of these cells to overcome the immunosuppressive tumor microenvironment (TME). We know from the clinical success of checkpoint inhibitor antibody therapy that the TME can be manipulated, allowing for both dormant and nascent T-cell responses to execute antitumor effects following

intervention (21), although not all solid tumors respond to checkpoint inhibition. Currently, the standard-of-care for use of CAR-T cell therapy is as a last line of defense; however, partnering CAR-T cells with other immunotherapeutics may be necessary to achieve efficacy in solid tumors. The remainder of this review will focus on the myriad strategies being investigated to enhance CAR-T cell efficacy in solid tumors.

**ENHANCING RESPONSES**

Productive T-cell responses require the convergence of three discrete signaling events. The first event is the signaling that takes place directly through TCR binding to its cognate peptide-MHC. The second event is the binding of costimulatory molecules, such as CD28, to their respective ligands on the opposing cell. The third event is signaling through cytokines, secreted factors that affect the fate of the cell, producing both stimulatory and inhibitory effects. As CARs already contain elements of the first and second signaling events, investigators have been manipulating stimulatory cytokines to enhance CAR-T cell efficacy.

To mitigate graft rejection, the current clinical protocol for T-cell based therapies is to use autologous T-cells. White blood cells are collected through leukapheresis, the whole T-cell population, including both $CD4^+$ and $CD8^+$ T cells, is then selected out, and the T cells are then cultured *ex vivo* (**Fig. 3**). During this time, exogenous cytokine supplementation is critical for T-cell proliferation, survival, and differentiation, and while there is some overlap with regards to cytokine function, the specific cytokines used can greatly impact the fate and function of CAR-T cells.

T cells differentiate into roughly four subsets: naïve, central memory, effector memory, and $CD45RA^+$ effector memory T cells. Interestingly, while the overall proliferation rate and differentiation of T cells are comparable when the cytokines IL-2, IL-7, or IL-15 are used, there is a noticeable difference in *in vivo* cytolytic function. T cells that were cultured with IL-2 exhibited poor antitumor efficacy compared to IL-7 and IL-15 (22). In that study, the authors utilized a CAR design that incorporated a CD27 costimulatory domain. There is an inverse relationship between IL-2 receptor signaling and CD27 expression (23), but it is unclear if this lack of *in vivo* efficacy is specific to this CAR design. This is reflective of the CAR field more broadly, where all aspects of the CAR design impact CAR and T-cell function and these characteristics must be determined through empirical optimization. Indeed, the proximity of the epitope in relation to the cell membrane surface (24), the source (IgG, CD8α, or CD28) of the hinge that links the scFv with the transmembrane domain, and the origin (CD8α or CD28) of the transmembrane domain itself, all affect the performance and antitumor efficacy of the CAR (25,26).

Although IL-2 was not as effective in the CAR utilizing a CD27 costimulatory domain, many investigators utilize IL-2 in their culture methods and demonstrate effective *in vivo* cytotoxicity. In fact, the MAGE-A3-specific engineered T cells described above were dosed concomitantly with IL-2 (4). Unfortunately, IL-2 is so ubiquitous in the function of both transferred and endogenous inflammatory and suppressive T cells that toxicity is a major limitation to its use. A creative approach to avoid unwanted IL-2 toxicity employs an engineered IL-2 receptor that recognizes an IL-2 ortholog. Sockolosky et al. developed a receptor-ligand pair that functions similarly to natural IL-2Rβ and IL-2, respectively, but does not bind endogenous IL-2. This modified receptor

can be transduced into T cells, and the IL-2 ortholog can be administered to promote specific T-cell proliferation and antitumor efficacy comparable to wild-type IL-2 signaling (27).

An alternative approach to supplementing with exogenous cytokines is to encode cytokines directly into the CAR construct. Adachi et al. generated CARs targeting human CD20 or fluorescein isothiocyanate (FITC), a fluorescent molecular label, providing a more generalizable comparator. The CAR constructs were designed to induce the engineered T cells to express IL-7 and CCL19, two molecules critical for the maintenance of the T-cell zone in lymph nodes. Moreover, these signaling molecules are important for recruiting antigen-presenting dendritic cells (DCs), so encoding these molecules into CAR-T cells allows for tumor-infiltrating CAR-T cells to establish lymphoid-like structures within the tumor, enhancing the endogenous immune response (28). The complete regression of tumor in their *in vivo* model when treated with CAR-T cells expressing IL-7 and CCL19 versus conventional CAR-T cells suggests that CAR-T cell-mediated tumor cell death can induce subsequent release of tumor antigens, antigen acquisition and presentation by the recruited DCs, and induction of endogenous T-cell responses that can enhance the antitumor effect, a mechanism known as epitope spreading. Induction of epitope spreading may become an important aspect of CAR-T cell therapy because it can sustain antitumor efficacy regardless of CAR-targeted antigen loss or poor persistence of CAR-T cells.

In the context of that work, the potential priming of new immune responses through epitope spreading suggests that this strategy may work synergistically with CTLA-4-directed checkpoint inhibitor therapy, while PD-1 antagonism may help to prolong the lifespan of the CAR-T cells. The natural life cycle of T cells involves activation, clonal expansion, execution of effector function, and then exhaustion and contraction. This cycle is dependent on the intensity of the stimulus,

and CAR-T cells that are strongly activated in the presence of antigen can undergo activation-induced cell death (AICD). Gargett et al. demonstrate in a model targeting the neuroblastoma antigen GD2, that interaction with antigen from the tumor or antibody-mediated cross-linking of CARs resulted in increased expression of PD-1 and engagement with its ligand, PD-L1, resulting in AICD. Intervention with anti-PD-1 antibody to block this signal abrogated AICD (29). In fact, this finding allowed them to amend an ongoing clinical trial (NCT01822652) to include concomitant administration of pembrolizumab (Keytruda, Merck) with their GD2-directed CAR-T cells. Rafiq et al. went one step further and designed CAR-T cells that also secrete a PD-1-blocking scFv, allowing for combinatorial therapies to be administered in a single drug (30). Other attempts to prevent CAR-T cell death have been accomplished by blocking pro-apoptotic signaling pathways commonly utilized by T cells, including Fas-FasL-mediated signaling, death receptor 5 (DR5), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In murine studies, CAR-T cell persistence *in vivo* is attenuated by the upregulation of these pro-apoptotic pathways and blockade of these pathways promotes survival. More importantly, using a defined TCR demonstrated that this pro-death phenotype is enhanced by signaling through the TCR as well as the CAR, indicating that over-stimulation of the T cell is detrimental to long-term survival and antitumor efficacy (31).

While staving off cell death to promote persistence is a critical component to effective CAR-T cell therapy, first and foremost, the cells need to be exemplary killers. There have been two molecular approaches utilized by multiple investigators to make cells resistant to tumor-mediated immunosuppression: IL-12-secreting and IL-18-secreting CAR-T cells. The reason why these two cytokines, in particular, are chosen, stems from their ability to stimulate the

production of interferon-γ (IFNγ), a critical signaling molecule for T cells (32). Due to its potency at stimulating both T and NK cells, it was originally thought that dosing with IL-12 as a single agent could be efficacious in the treatment of solid tumors; however, its lethal toxicity in a Phase II trial terminated its therapeutic use. It is thought that local production of IL-12 by CAR-T cells, rather than systemic administration, may produce less toxicity. These IL-12-producing CAR-T cells, nicknamed “armored CAR T-cells," can alter the TME by depleting the highly suppressive tumor-associated macrophages through Fas-FasL interactions. Moreover, IL-12-secreting CAR-T cells are not inhibited by the PD-1-PD-L1 signaling axis (33). While impressive, it is unclear if these suppression-resistant armored CAR-T cells pose additional toxicity risk due to their persistence. It should be noted that in a clinical trial (NCT02498912) targeting MUC16 on ovarian tumors with IL-12-secreting CAR-T cells, the investigators have also encoded a truncated epidermal growth factor receptor (EGFRt) that lacks both ligand-binding and enzymatic activity but allows for these cells to be eliminated with cetuximab.

The alternative approach to IL-12 is to armor CAR-T cells by encoding the other IFNγ-inducing cytokine, IL-18. As such, many of the antitumor results are similar, although there are some important distinctions that should be noted. IL-18-secreting CAR-T cells were also capable of remodeling the TME, albeit by increasing the number of effector cells, rather than depleting the number of suppressive cells. In one murine study using the thymoma cell line EL4 engineered to express human CD19, IL-18-secreting CAR-T cells trafficked to the bone marrow, where these tumor cells engraft after intravenous administration, whereas conventional CD19-directed CAR-T cells did not. These armored CAR-T cells promoted the expansion of NK cells, NKT cells, DCs, and endogenous $CD8^+$ T cells. There was also more pro-inflammatory M1 macrophages present

(34). IL-18 functions in tandem with TCR signaling, so it was important to determine if IL-18-mediated antitumor efficacy was the result of non-specific, endogenous TCR activation of the CAR-T cells or if the CAR was sufficient to produce the observed IL-18 response. Hu et al. used TCR knock-out CAR-T cells to demonstrate that the IL-18-mediated effects were maintained by the signaling through the CAR (35). To improve safety, they placed IL-18 under the control of the nuclear factor of activated T-cells (NFAT) promoter, which becomes transcriptionally active after TCR, or in this instance, CAR signaling. Therefore, IL-18 is actively produced only after CAR signaling commences upon binding of its target antigen. Mechanistically, it was discovered that these inducible IL-18 CAR-T cells promoted a more cytolytic $CD8^+$ T-cell response by inducing a T-$bet^{high}$ $FoxO1^{low}$ phenotype, whereas the opposite transcription factor signature promotes memory $CD8^+$ T-cells (36).

The cytokine milieu plays a critical role in shaping the immune response, and while IL-12 and IL-18 promote proliferation, survival, and effector functions of CAR-T cells, there are many tumor-secreted cytokines that have the opposite effect. Most notable is transforming growth factor β (TGFβ), a critical cytokine in the differentiation of suppressive regulatory T cells (Tregs) and maintenance of T-cell homeostasis (37). Rather than allowing the TGFβ-enriched TME to exert immunosuppressive pressure on CAR-T cells, investigators are developing ways to block the signal by creating a dominant-negative receptor or exploiting this signal by coupling the extracellular domain of the TGFβ receptor to a stimulatory intracellular domain. In the latter example, Sukumaran et al. created a system that required unique signaling events to trigger the three discrete signals required to produce a productive T-cell response. Signal 1, the TCR signal, was generated using a first-generation CAR containing the CD3ζ endodomain fused to a scFv

recognizing the prostate stem cell antigen (PSCA). The second signal, the costimulatory signal, was created by fusing the extracellular TGFβRII domain to the 4-1BB signaling domain. The third signal, the cytokine signal, was generated by fusing the extracellular receptor domain for the suppressive cytokine IL-4, which is also generated by some tumors, to the intracellular IL-7Rα domain (38). Although there are technical limitations to this approach due to the requirement for multiple constructs encoding the three elements, this proof-of-concept for a logic-gated system to promote specificity and safety of split domain CARs requiring independent stimuli appears to be a viable option. It is, however, unclear if the signal 3 component is redundant. The IL-4/IL-7 switch receptor might enhance stimulatory signals, although it is unclear if endogenous cytokines would be sufficient if the T cells expressed only the PSCA CAR and TGFβRII-4-1BB receptors. An alternative approach is to abrogate TGFβ signaling by incorporating a dominant negative receptor. Using a CAR targeting the prostate-specific membrane antigen (PSMA), Kloss et al. created a T cell that expresses both the CAR and the dominant negative TGFβ receptor. Insensitivity to TGFβ promoted a significant increase in proliferative capacity relative to conventional CAR-T cells (39), suggesting that the lack of persistence observed in CAR-T cell-treated solid tumors stems from TME-mediated immune suppression and that the potential to achieve the persistence observed in CD19-directed CAR-T cells is possible with appropriate immune cell modulation.

**GENE EDITING APPROACHES**

The ability to enhance CAR-T cell efficacy is incumbent on how the T cells are transformed into CAR-T cells. To date, the method of gene delivery has used lentiviral or γ-retroviral vectors. The

two FDA approved CD19 CAR-T cell therapies, Kymriah and Yescarta, use lentiviral or retroviral delivery, respectively. While these methods for gene delivery into T cells are effective, genome integration is uncontrolled. Lentiviral vectors adopt their transcriptional machinery from the human immunodeficiency virus (HIV), and while the exact location of integration cannot be controlled, leading to potentially deleterious effects, HIV is known to integrate at transcriptionally active sites (40). In a remarkable case study of a seventy-eight-year-old patient with CLL treated with CTL019, the therapy that would become Kymriah, it was found that a single CAR-T cell clone was responsible for mediating the bulk of the antitumor response (41). In this instance, the lentiviral vector integrated into intron 9 of the methylcytosine dioxygenase *TET2* gene of a single T-cell clone, disrupting its function. The patient's second *TET2* allele possessed a hypomorphic mutation resulting in complete loss of TET2 function in the clone. Interestingly, the *TET2*-disrupted clone was able to expand significantly upon repeated antigen stimulation compared to CAR T-cells with intact *TET2*. These CAR T-cells also had increased expression of the cytolytic effector molecules perforin and granzyme B, consistent with the increased cytotoxic effector function observed in these cells. This data suggests that strategic genomic insertion of the CAR can have a broad phenotypic impact on the T cell and dramatically alter therapeutic efficacy.

Gene editing techniques need not be as stochastic as viral vectors. Numerous approaches allow for site-specific integration by creating double-strand breaks that can be repaired with the insertion of a transgene. Most notable among these techniques is CRISPR/Cas9. Considering that the CAR is a surrogate for the TCR, the most obvious location to target is the TCR locus. Eyquem et al. created a CRISPR system to insert the CAR construct within the T-cell receptor α chain

constant (*TRAC*) locus, resulting in superior antitumor efficacy (42). To elucidate the functional differences between conventional and *TRAC*-CAR-T cells, lower and lower doses of cells were administered, with *TRAC*-CAR-T cells maintaining therapeutic efficacy, whereas conventional retrovirus-transduced CAR-T cells were no better than controls. Site-specific insertion of the CAR into the *TRAC* locus prevents the acquisition of an exhausted phenotype, which is abrogated by the inclusion of a constitutive EF1α promoter, suggesting that transcriptional regulation in a site-dependent manner is important to maintain CAR-T cell potency.

Although there are benefits to using CRISPR/Cas9 gene editing, including the simplicity of design and ability to target multiple genes in a single cell, there is a high likelihood for off-target effects (43). Derived from xanthamonus bacteria, transcription activator-like effector nucleases (TALENs) allow for highly accurate and efficient gene editing. To instigate a double-strand break, a left and right TALE must be designed with the assistance of software that allows for any region of DNA to be targeted. A FokI nuclease domain is then added to both the left and right TALE domains, such that the two nucleases meet at the same location on the DNA and instigate cleavage (44). By creating two double-strand breaks within the same chromosome, the cell can utilize homology-directed repair, allowing for the precise insertion of specific genes of interest. TALENs have been used to create site-specific alterations to the TCRα and β loci. Insertion of a flu-specific TCR into primary human T cells resulted in a five-fold increase in IFNγ and an 18-fold increase in IL-2 production compared to conventionally transduced cells (45). This corroborates the improved effector function seen in the CRISPR-edited *TRAC*-CARs. In CAR-T cells, TALENs have been used to selectively knock out specific loci. To treat two infants with relapsed and refractory B-ALL, the difficulty in manufacturing autologous cellular products required an alternative

strategy. HLA-mismatched donor cells were transduced with CD19 CAR-encoding lentivirus followed by the use of TALENs to delete both the TRAC locus and CD52, the antigen targeted by the transplantation drug alemtuzumab (46). Both infants responded to treatment with these cells and were in remission, although there was graft-versus-host disease noted in both cases, most likely caused by residual $TCR^+$ CAR-T cells. Nonetheless, this suggests that as a proof-of-concept, universal CAR-T cells can be implemented using modern gene-targeting approaches with clinically-manageable toxicity risks.

**CONCLUSIONS**

Given the difficulty in treating solid tumors with CAR-T cells, the success witnessed in CD19-directed CAR-T cell therapy may appear serendipitous. There is potentially some truth to this, as CAR-T cell therapy to treat T-cell acute lymphoblastic leukemia (T-ALL) has not been developed concurrently with B-ALL CAR-T cell therapy, given the risk for fratricide when targeting T-cell antigens expressed in not only T-ALL cells but also in the CAR-T cells themselves. Experience with CD19-directed CAR-T cell therapy has encouraged the CAR-T cell community to confront the problems associated with expanding this therapy to other tumor types. In fact, in the case of T-ALL, Cooper et al. have created a CAR that targets the T-cell antigen CD7 and, to avoid CAR-T cell fratricide, have used CRISPR/Cas9 to delete CD7 in the CAR-T cells (47). The necessary strategies to create more effective CAR-T cell therapies are being developed and this review has outlined some of the approaches investigators are taking to enhance the efficacy of these cells both within CD19-targeted therapy and beyond. Although a one-size-fits-all approach is ideal, and investigators are seeking a cytokine signaling pathway that is a panacea for the limitations of

treating solid tumors and unleashing the full potential of CAR-T cells, the reality is that each cancer is unique and will ultimately require unique approaches to effectively eradicate disease using CAR-T cells.

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**FIGURES**

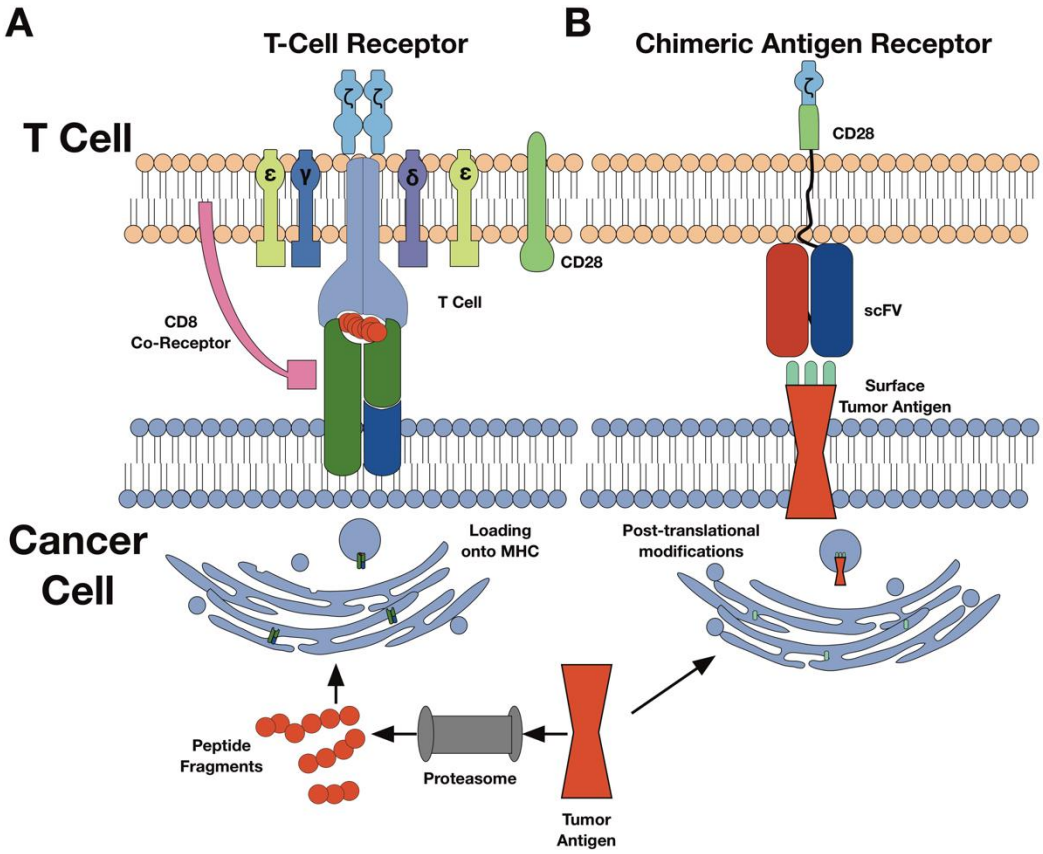


**Figure 1. Comparison of T-Cell Receptors and Chimeric Antigen Receptors**

**A**) Virtually all cells express the Major Histocompatibility Complex (MHC) class I molecules, including cancer cells. Antigens produced by the cancer cell go through a processing mechanism in the proteasome producing short (8-15 amino acid) peptide fragments that are loaded onto MHC class I molecules and presented on the surface of the cell. This peptide-MHC complex is recognized by the T-cell receptor (TCR), which is composed of an α and β subunit that forms the binding domain, surrounded by the CD3 molecules δ, ϵ, γ, and ζ. $CD8^+$ T cells recognize class I MHC and the CD8 co-receptor binds to the MHC class I molecule, stabilizing the interaction. This is considered Signal 1 of a productive T-cell response. Signal 1 by itself leads to anergy, a state of functional inactivation. An additional signal, through the co-stimulatory receptor CD28, known as Signal 2, is required to fully activate the T cell. **B**) Tumor antigens that are trafficked to the cell surface after post-translational modifications can be recognized by a chimeric antigen receptor (CAR). The antigen binding domains of the heavy and light chains from an antibody are fused with a short, flexible peptide linker, creating a scFv. This portion of the molecule allows the CAR to recognize a tumor antigen independent of presentation by MHC molecules. The Signal 1 and Signal 2 components of the TCR complex are then located intracellularly and are attached to the scFv through hinge and transmembrane domains. Originally, CARs contained only a CD3ζ intracellular signaling domain, and this lack of Signal 2 resulted in anergic-like T cells. The addition of CD28 or 4-1BB costimulatory domains provided the Signal 2 required for productive T-cell responses. Whether signaling through an endogenous TCR or a CAR, the result is the same T cell-mediated killing of the target cell.

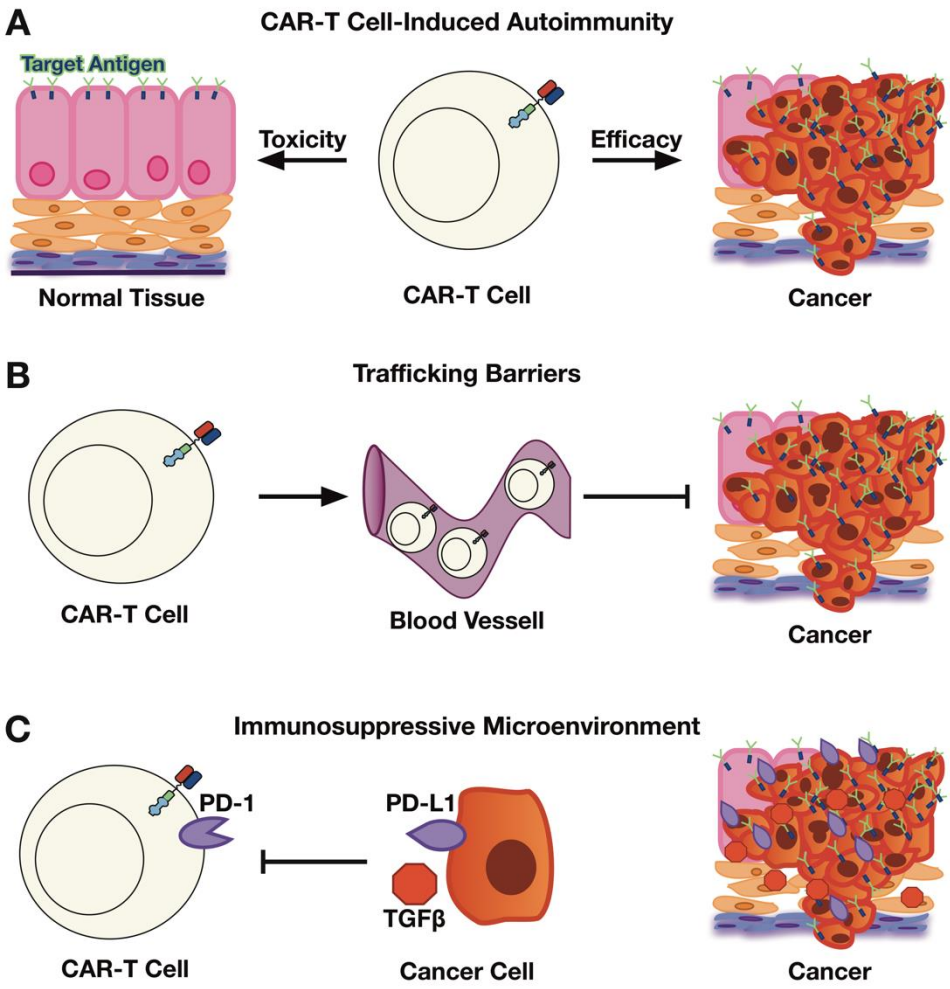


**Figure 2. Immunotherapeutic Barriers to Treating Solid Tumors**

The success of CAR-T cell therapy for the treatment of hematological malignancies has resulted in FDA approval of two, novel immunotherapeutics; however, solid tumors pose additional barriers that have yet to be overcome in the successful implementation of CAR-T cell therapy. **A**) The first barrier is identifying appropriate target antigens that demonstrate differential expression between normal tissue and tumor. Indeed, the CD19-directed CAR T-cell therapies target healthy B-cells; however, this autoimmunity can be successfully treated through IVIG administration. CAR-T cell-induced autoimmunity in solid organs can lead to life threatening morbidities and even death from T-cell mediated destruction of the organ. Thus, finding appropriate antigens is a critical first step in identifying a successful therapy. The second limitation is trafficking of the CAR-T cells to the tumor. **B**) Abnormal homing molecule (adhesion molecules and chemokines) expression and tumor vasculature can create conditions that make it difficult for T cells to extravasate into tumor tissue and execute their effector function. **C**) The final barrier, once CAR-T cells reach the tumor, is to overcome the tumor's immunosuppressive microenvironment. Solid tumors secrete immunosuppressive cytokines, such as TGFβ. Moreover, IFNγ secreted by activated T-cells, including CAR-T cells, can induce expression of PD-L1 in the tumor, which is one of the critical inhibitory immune checkpoints. Blocking these inhibitory pathways might be important for successful CAR-T cell responses in solid tumors.

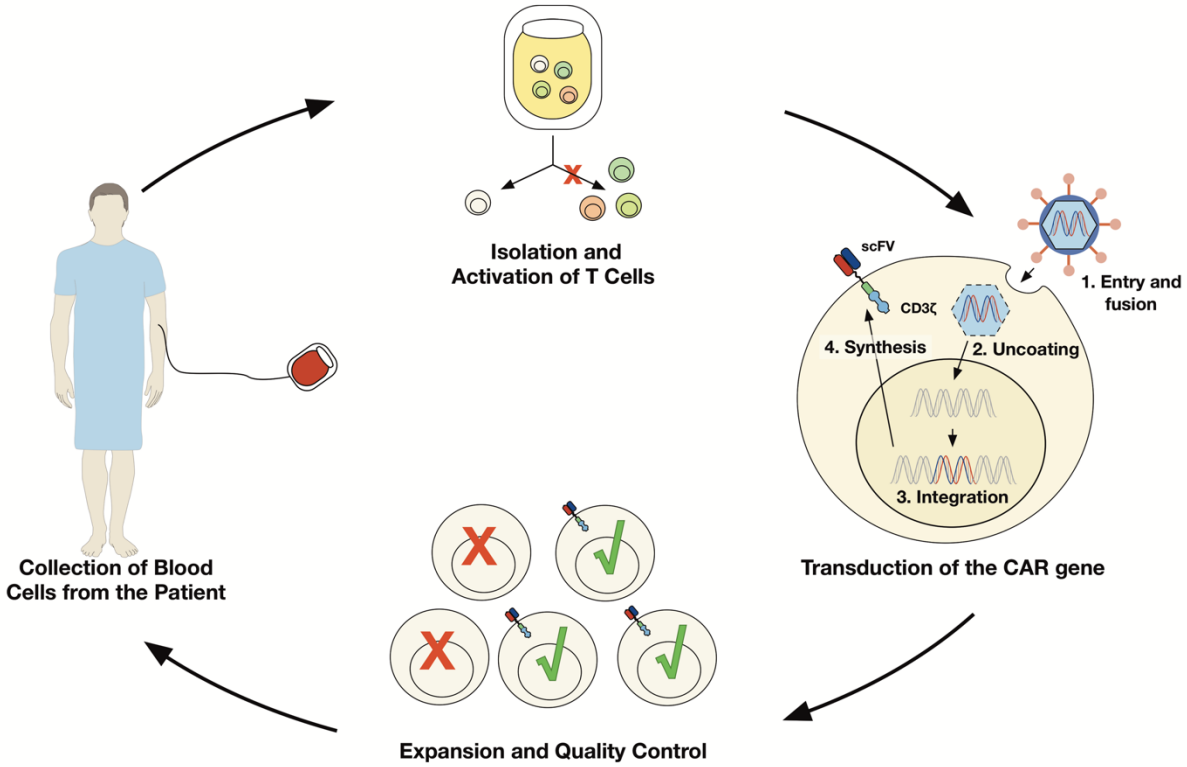


**Figure 3. Clinical Life Cycle of a CAR-T Cell**

Patients who are relapsed or refractory are considered candidates for CAR-T cell therapy. To avoid graft rejection, the patient's T cells are used to create the product. White blood cells are isolated from the blood by leukapheresis. T cells are then isolated from the population and the cells are activated by stimulation with antibodies against CD3 and CD28. This begins the proliferation phase of the *ex vivo* culture process and the cells are next transduced using either a lentivirus or a γ-retrovirus. The virus integrates into the host-cell genome and the CAR is produced by the cell and trafficked to the surface. The cells are then expanded to have suitable numbers for infusion and quality control measures are taken to ensure that the product is safe and free from contaminants. The CAR-T cells are ready for infusion back into the patient to eliminate their cancer.